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Low thermodynamic but high kinetic stability of an antifreeze protein from *Rhagium mordax*

Dennis S. Friis,* Johannes L. Johnsen, Erlend Kristiansen, Peter Westh, and Hans Ramløv

Department of Science, Systems and Models, Roskilde University, DK-4000 Roskilde, Denmark

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Abstract: The equilibrium heat stability and the kinetic heat tolerance of a recombinant antifreeze protein (AFP) from the beetle *Rhagium mordax* (RmAFP1) are studied through differential scanning calorimetry and circular dichroism spectroscopy. In contrast to other insect AFPs studied with this respect, the RmAFP1 has only one disulfide bridge. The melting temperature, T_m, of the protein is determined to be 28.5°C (pH 7.4), which is much lower than most of those reported for AFPs or globular proteins in general. Despite its low melting temperature, both biophysical and activity measurements show that the protein almost completely refolds into the native state after repeated exposure of 70°C. RmAFP1 thus appears to be kinetically stable even far above its melting temperature. Thermodynamically, the insect AFPs seem to be dividable in three groups, relating to their content of disulfide bridges and widths of the ice binding motifs; high melting temperature AFPs (high disulfide content, TxT motifs), low melting temperature but high refolding capability AFPs (one disulfide bridge, TxTxTxT motifs) and irreversibly unfolded AFPs at low temperatures (no disulfide bridges, TxTxTxTxT motifs). The property of being able to cope with high temperature exposures may appear peculiar for proteins which strictly have their effect at subzero temperatures. Different aspects of this are discussed.

Keywords: antifreeze protein; thermodynamics; differential scanning calorimetry; circular dichroism; refolding; protein melting temperature

Introduction

Antifreeze proteins (AFPs) are found in a wide variety of cold-tolerant ectothermic organisms.[1–4] These proteins are a structurally diverse group defined by their shared ability to cause a separation of the melting- and freezing point of ice crystals in solution. The phenomenon is termed thermal hysteresis or the antifreeze activity.[5] AFPs presumably serve as a protective measure against lethal internal ice formation.[6] The first AFPs studied, originated from fish living in Polar Regions.[5,7] These AFPs could lower the ice crystal growth temperature on the order of 1–2°C below the melting point of the body fluids.[2] There has been a growing interest in these proteins since the discovery of "hyperactive" AFPs from insects, in which haemolymph an antifreeze activity of up to 9°C has been observed,[8] both because of their unique ability and their potential technological use.

The stability of proteins may be understood in two different ways.[9] The thermodynamic stability is defined with respect to an equilibrium between the native, biologically active (N-) form, and an ensemble of non-native conformations sometimes called

Abbreviations: AFP, antifreeze protein; CD, circular dichroism; DSC, differential scanning calorimetry; T_m, melting temperature.

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*Correspondence to: Dennis S. Friis, Department of Science, Systems and Models, Roskilde University, Universitetsvej 1, P.O. Box 260, Building 18.1, DK-4000 Roskilde, Denmark.
E-mail: dsf@ruc.dk

Table I. *Melting Temperature and Other Related Physical Parameters of Various AFPs*

Species	Isoform/name	Structure	Size (kDa)	T_m (°C)	Cysteines	Ref
Pseudopleuronectes americanus	17 kDa	α-helix (Type I)	16.7	55	0	19
	AFP9	α-helix (Type I)	4.3	18	0	43
Macrozoarces americanus	HPLC12 (PDB: 1MSI)	Globular (Type III)	7.4	47	0	13
	HPLC12 (PDB: 1HG7)	Globular (Type III)	7	50	0	20
	HPLC6	Globular (Type III)	6.9	40	0	25
	rAFP (HPLC6, recombinant)	Globular (Type III)	7.2	44	0	25
Austrolycicthys brachycephalus	AB1	Globular (Type III)	6.8	43	0	26
Dendroides Canadensis	DAFP-4	β-helical	7.3	84	14	21
Tenebrio Molitor	YL-1 (PDB: 1EZG)	β-helical	8.4	66	16	22
Rhagium mordax	RmAFP1	β-helical	12.5	29	2	This work

the unfolded (U-) form. Alternatively, one may assess protein stability from a kinetic point of view and describe the rate by which the N-form is lost to an irreversibly inactivated (I-) form. One of the most common decay routes for irreversible transition is aggregation. Substances that do not aggregate are called colloidally stable. Proteins that avoid aggregation usually have the ability to refold, thus, proteins' ability to refold are therefore frequently referred to as their colloidal stability.[10] Lumry and Eyring[11] introduced an interpretation which links the two approaches and has been successfully used in countless later studies. In the simplest version the Lumry-Eyring model stipulates the reaction course

$$N \underset{k_-}{\overset{k_+}{\rightleftarrows}} U \xrightarrow{k_{ag}} I \quad (1)$$

where k_-, k_+, and k_{ag} are the first order rate constants for the respective transitions. The consequence of Eq (1) is that the native protein must go through the reversible unfolding step before it is prone to irreversible inactivation. In Eq (1), the thermodynamic stability is defined by the equilibrium constant $K = k_+/k_-$ while the colloidal stability is dependent on all three rate constants. At temperatures above T_m (i.e., for $K > 1$); however, the colloidal stability is essentially governed by k_{ag}.

The present knowledge of the stability of AFPs is superficial, and to our knowledge only two experimental studies[12,13] have focused specifically on this topic. However, several observations regarding the thermostabilities of various AFPs have been reported and are presented in Table I. All T_m presented are obtained at pH values between 7.0 and 8.0. In addition, some phenomenological findings are reported on AFP stability; The β-helical AFP from the beetle *Microdera punctipennis* (MpAFP698, 13.9 kDa, 18 cysteines) maintained almost all its activity after 5 min exposure to 100°C,[12] indicating a high melting temperature or a high degree of refolding. The β-helical AFP from the moth *Campaea perlata* (mixture of AFPs, 3.4–8.3 kDa, 0–1 cysteines) lost all activity after 2 h at room temperature (22°C).[14] The β-helical AFP from the beetle *Rhagium inquisitor* (RiAFP, 12.5–12.8 kDa, 2 cysteines, PDB: 4DT5), a species closely related to the AFP of this study from *Rhagium mordax*, maintained activity after 5 min exposure to 70°C.[15] The β-helical AFP from the ryegrass *Lolium perenne* (LpAFP, 13.5 kDa, 0 cysteines, PDB: 3ULT) was active after 5 min exposure to 95°C; however, circular dichroism (CD) analysis showed that not all of the protein refolds into a native state after exposures to 60°C (where complete unfolding was observed).[16] The AFP from the springtail *Hypogasttura harveyi* consisting of stacked antiparallel helices[3] (sfAFP, 6.5 kDa, 4 cysteines, PDB: 2PNE) lost activity (antifreeze activity was reduced from 3.5 to 0.2°C) after exposure to 22°C over night (supplementary materiel of Ref. 17). The AFP from the bacterium *Flavobacterium xanthum* (IAM12026, 59 kDa) lost activity after exposure to 60°C for 1 h.[18] The stated protein sizes are of those used in the studies (often a recombinant protein with a small tag) and not that of the wild type.

The heat tolerances of the AFPs are thus quite diverse not only overall but also among the fish or insect AFPs. The varying stabilities in insect AFPs are, at least in part, connected to the varying number of cysteines bridges in the proteins, which causes a more or less rigid and heat stable structure. However, the proteins' ability to cope with high temperatures is also dependent on the colloidal stability of the unfolded molecule.

Several research groups have reported high refolding abilities of AFPs after heat denaturation,[13,19–22] but only in two studies have activity measurements been carried out after an observed refolding to test if the protein had undergone a reverse transition into the native, active form.[19,21] For the *P. americanus* 17 kDa AFP, it was observed that the activity after being exposed to room temperature (22°C) was not as high as expected on the basis of CD-measurement of refolding. The studied AFP was a dimer, and the loss of activity might have been caused by a loss of tertiary or quaternary structure.[19] For the *D. canadensis* DAFP-4 no

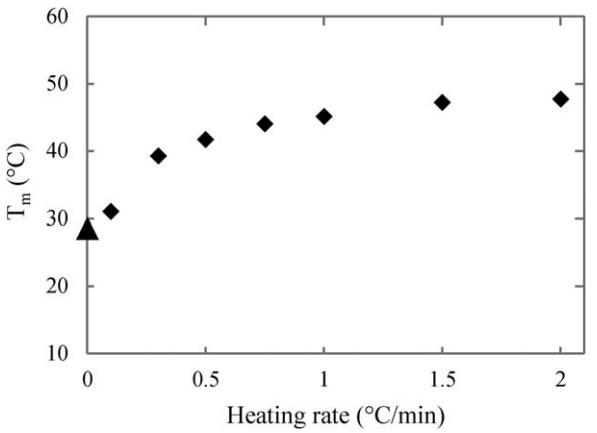

Figure 1. Measurements of T_{mApp}. The diamonds represents T_{mApp} determined at different heating rates by DSC and the triangle represents the T_m determined by CD.

change in activity was observed after 5 min exposures of up to 100°C, well above the T_m of 84°C.[21] For the *M. punctipennis* MpAFP698 activity was maintained after 5 min at 100°C. However, whether the protein was unfolded at this temperature and subsequently refolded to an active form upon cooling was not established.[12] So far only the DAFP-4 has been documented to maintain its activity after exposures above its determined melting temperature.

During purification processes of a novel recombinant AFP from the beetle *R. mordax* (RmAFP1), it was observed that the antifreeze activity was maintained after exposures to 60°C for 10 min. In this study, we investigated both the thermodynamic- and colloidal-stability of this protein. The RmAFP1 differs from DAFP-4 as it contains only one disulfide bridge and has a broad TxTxTxT ice binding motif, contrary to the narrow TxT motif of DAFP-4. The T_m of RmAFP1 was determined using CD and differential scanning calorimetry (DSC). Through DSC the refolding capabilities of RmAFP1 were quantified by successive heat cycles. The refolding rate, k_-, was determined in the interval of 1–20°C.

Results

Protein melting temperature

As the oscillation of the calorimetric trace on the DSC reflects the enthalpy of the protein unfolding, the apparent melting temperature, T_{mApp}, was defined as the temperature where this signal was strongest, for example, at 1°C/min the signal peaks at 45.1°C [see Fig. 3(A)] which is thus the T_{mApp} at this heating rate. The T_{mApp} determined at different heating rates are shown in Figure 1.

The different heating rates resulted in T_{mApp} spanning from 31.0°C at 0.1°C/min to 47.7°C at 2.0°C/min. This suggests a profound dependence of the observed T_m on the heating rate. Thus, the T_{mApp} for heating rates typically used in DSC experiments (0.5–1°C/min) were about 15°C higher than the extrapolated value for the heating rate of zero. To circumvent the influence of the heating rate, the protein T_m was also determined using CD spectroscopy with extensive equilibration times at test temperature prior each measurement. As the CD spectrum of the folded protein has its global minimum at 218 nm, the degree of unfolding was calculated from average standardized $mdeg_{218}$ values at different temperatures, which is shown in Figure 2.

The degree of unfolding showed convergence with a sigmoid course (c.f. the materials and methods section), with the critical interval spanning 20–40°C. At high temperatures (50°C and above) a small decline in the unfolded fraction was observed. This could either be caused by heat induced protein loss or if the CD signal had a slight temperature dependent drop. By data transformation and linear regression of the data points in the interval of 10–39°C ($n = 6$, $R^2 = 0.996$) the constants a and b in Eq. (6) were derived and T_m was calculated to be 28.5°C. This value is plotted in Figure 1, where it lines up well with extrapolations of the data from the DSC to heating rates approaching 0°C/min.

Refolding ability

Through DSC scans we obtained an average enthalpy of unfolding (ΔH_{unfold}) of 266 kJ/mol (±SEM 9.6, $n = 6$) for RmAFP1. To assess the refolding abilities of the protein, 10 successive heating scans from 1 to 60°C, 70 or 80°C, respectively, were made with 45 min of equilibration at 1°C between each run [Fig. 3(A)]. The resultant ΔHs were standardized according to the first run [Fig. 3(B)].

The thermal profile showed little variation among the 1–60°C heating scans [Fig. 3(A)]. ΔH

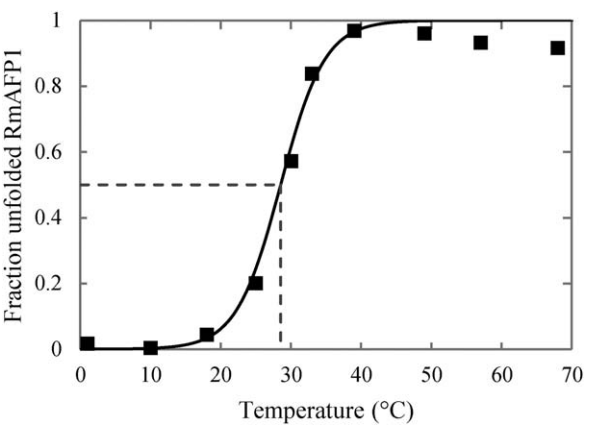

Figure 2. Fraction of unfolded RmAFP1 standardized from the extreme values at wavelength 218 nm. Squares represent data points and the line is the best fit of Eq. (6). T_m (28.5°C) illustrated with a circle, is given as the temperature where the fraction of unfolded (and folded) protein equals 0.5.

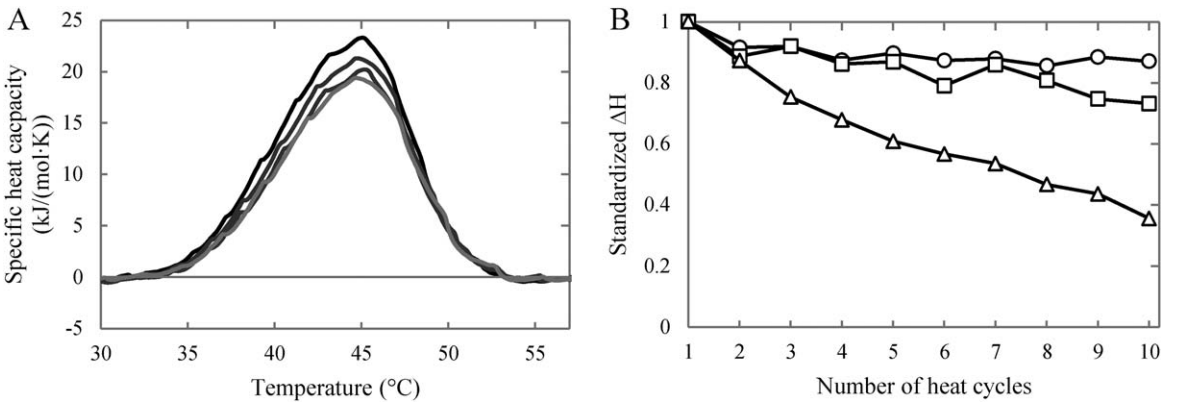

Figure 3. Thermal profiles of RmAFP1. A: ΔH_{unfold} of RmAFP1 in 10 successive heat cycles from 1 to 60°C [only heat cycle 1 (black), 4, 7, and 10 (light gray) are shown]. B: The relative changes of ΔH_{unfold} in 10 succeeding heat cycles of 1–60°C (circles), 1–70°C (squares), and 1–80°C (triangles), respectively. The values are standardized to the ΔH_{unfold} of the first run.

showed an average decline of 1.0% of its original value between each run, indicating a near complete refolding to the native state. For cycles from 1–70°C to 1–80°C, ΔH decreased on the average of 2.4 and 6.5% per run [Fig. 3(B)], respectively, indicating a moderate irreversible unfolding at these temperatures. Thus, the total losses of ΔH during the 10 scans were 10, 24, and 65%, for the samples heated to 60, 70, and 80°C, respectively.

To verify a correct refolding, that is, an active protein, the heat-treated samples were retrieved and the antifreeze activity measured and compared to an untreated sample. Before heat treatment the activity was measured to 2.325°C (±SEM 0.086, $n = 10$), and after 10 cycles the activities were measured to 1.955°C (±SEM 0.066, $n = 10$), 2.033°C (±SEM 0.041, $n = 10$), and 1.581°C (±SEM 0.101, $n = 10$) for the samples heated 10 times from 1 to 60, 70 and 80°C, respectively. Assuming that the percentage drop in ΔH corresponds to the percentage loss of protein, the 10 successive heating scans to 60 and 70°C had caused a drop in hysteresis roughly corresponding to the loss of protein, when comparing with an activity curve for RmAFP1 (not shown). Here, the expected activities after a protein loss of 10% and 24% were 2.1 and 1.8°C. However, samples from the 80°C scans show an activity much higher than the expected value of 0.5°C from the 65% protein loss.

Refolding rate

The refolding rates at various temperatures were assessed through CD spectroscopy. The samples were heated to 50°C for 12 min to completely denature the protein samples and then cooled to 1, 5, 10, 15, or 20°C, respectively. Here, the spectra were measured continuously until they were stable, that is, where structural equilibrium had been obtained. Figure 4(A) shows a subset of these spectra. Here, the spectra at the two extreme states are shown together with spectra after 4 and 10 min at 1 and 15°C, respectively. As evident from Figure 2, virtually all protein should be in its folded state at equilibrium at these temperatures. From Figure 4(A), it can be seen that the protein was almost fully refolded after only 10 min at 1°C, whereas at 15°C only a small fraction have refolded within the same

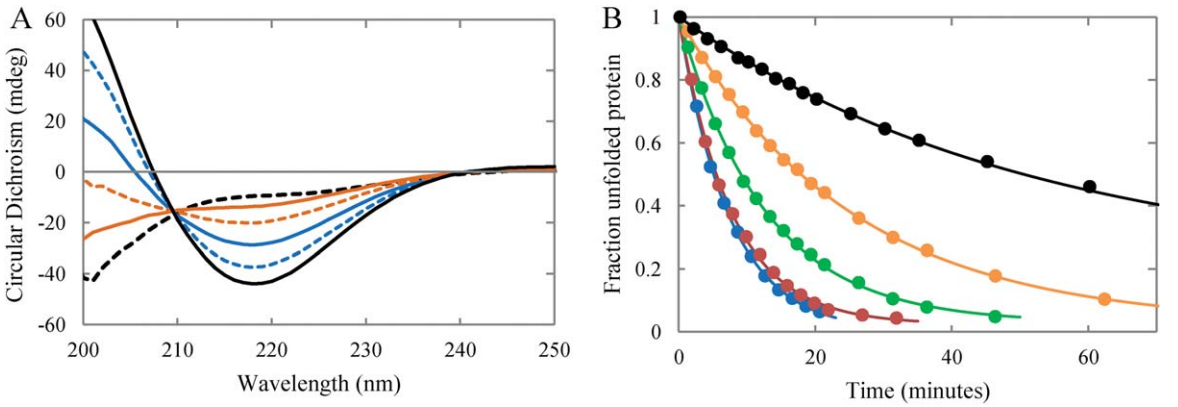

Figure 4. Refolding of RmAFP1. A: CD spectra for RmAFP1 in the unfolded state (dashed black line), folded state (solid black line) and 4 min (solid lines) and 10 min (dashed lines) after cooling from 50 to 1°C (blue lines) or to 15°C (orange lines), respectively. B: Change of the unfolded protein fraction after cooling the sample to different temperatures (blue: 1°C, red: 5°C, green: 10°C, orange: 15°C, and black: 20°C).

time period. Thus, the refolding rate of RmAFP1 increased as the temperature was lowered. The measured $mdeg_{218}$ were transformed to relative terms to reflect the fraction of unfolded protein in the sample. The change in the fraction of unfolded protein, F_u, over time at the five different temperatures is illustrated in Figure 4(B). The data points were adjusted with a time interval reflecting the magnitude the spectra had changed from the beginning of the cooling from 50°C to the first measurement was taken at the different temperatures.

Figure 4(B) shows an inverse relation between refolding rate and temperature in the interval 20–1°C. These data allowed for the determination of the reaction rate of folding, k_-, assuming that the amount of aggregated protein was zero as we observed almost total refolding of the protein after exposures to 60°C (c.f. Fig. 3). Hence, the total amount of protein, P_0, equals the sum of the unfolded (U) and folded (N) populations. The rate of change in concentration of the unfolded fraction may be written as

$$\frac{d[U]}{dt} = k_+[N] - k_-[U] \quad (2)$$

Substituting $[N]$ with $P_0 - [U]$ and rearranging the equation we get,

$$\frac{d[U]}{dt} = k_+P_0 - (k_- + k_+)[U] \quad (3)$$

The solution to this differential equation is

$$\frac{[U](t)}{P_0} = \frac{k_+ + k_- e^{-(k_+ + k_-)t}}{k_+ + k_-} \quad (4)$$

where $[U](t)/P_0$ corresponds to F_u, the fraction unfolded protein at time t. Values of k_- and k_+ were determined by fitting the data in Figure 4(B) to Eq. (4) using nonlinear regression. All data sets gave R^2 values > 0.99. Only those data points where $F_u > 0.05$ were included. The refolding rates, k_-, at 1, 5, 10, 15, and 20°C were found to be 8.14, 7.64, 4.63, 2.35, and 0.93 h^{-1}, respectively, shown in Figure 5.

Reliable k_+ values could not be obtained by this method, as the rates are very low at these temperatures, thus being very susceptible to the experimental noise. However, the k_+ was determined from $k_+ = k_-[U]/[N]$, where $[U]/[N]$ was obtained from Eq. (6), on the assumption that $[I] = 0$. The k_+ values corresponding to 1, 5, 10, 15, and 20°C were thus 0.0013, 0.0043, 0.0128, 0.0319, and 0.0617 h^{-1}, respectively. This result, i.e. that $k_- >> k_+$ at these temperatures, implies that unfolding may be neglected and, hence, that Eq. (5) may be reduced to

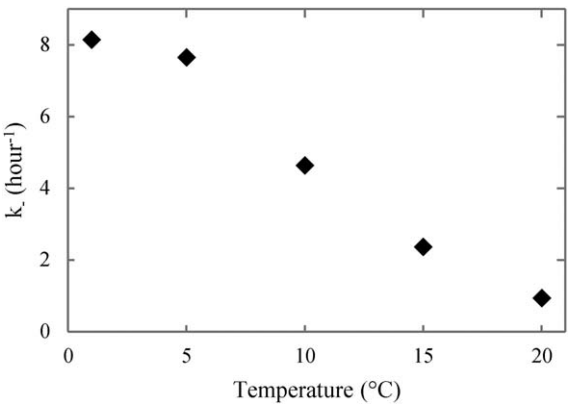

Figure 5. Rate of refolding. The refolding rates, *k*, versus temperature show an inverse relationship, which indicates negative apparent activation energy.

$[U](t)/P_0 = e^{-k-t}$, describing a reaction of exponential decay with k_- being the decay constant.

An increase in transition rate as the temperature is lowered indicates negative apparent activation energy. This may occur, for example, when a process relies on a complex set of elementary steps.[23] One way to assess whether a simple two state unfolding step [as suggested in Eq. (1)] was relevant for RmAFP1 was to perform a Van't Hoff analysis of the calorimetric trace[24] and hence obtain a model-dependent value of the transition enthalpy. Comparisons of this and the (model independent) calorimetric value of ΔH shed some light whether Eq. (1) provided a realistic description of the process. In our case, Van't Hoff analysis of the scans at 0.3°C/min suggested a ΔH of ~340 kJ/mol; that is, moderately larger than the calorimetric value of 266 kJ/mol. However, as the transition was not fully in equilibrium even during this slow heating rate (c.f. Fig. 1) this difference cannot be interpreted unequivocally with respect to possible intermediate states of unfolding.

Discussion

Low melting temperature of RmAFP1

The T_m of RmAFP1 at pH 7.4 was determined by CD spectroscopy to be 28.5°C. This method is widely used for this purpose,[20,21,25–28] and has the advantage over DSC that it circumvents the question of heating rate. With DSC the T_{mApp} was calculated to be 45.1°C at 1°C/min. This is 16.6°C above the T_m determined by CD. Even at the lowest heating rate achievable on the DSC, 0.1°C/min, we obtained a T_{mApp} 2.5°C above the 28.5°C. This phenomenon is linked to the protein's rate constants, and is more evident the lower the rate constant of unfolding. This issue has been addressed by Potekhin and Kovrigin[29] who present an equation allowing for the calculation of the maximum heating rate, V_{max}, to be used without diverging from equilibrium;

$$V_{max} = \frac{RT_m{}^2}{\Delta H(k_+(T_m)+k_-(T_m))^{-1}} \quad (5)$$

By extrapolating the k_- values obtained at 5–20°C to the T_m at 28.5°C ($R^2 = 0.981$, exponential regression) we obtain a k_- and k_+ of 0.00526 min^{-1}. Inserting these values in Eq. (5) the maximum heating rate for unbiased melting temperature determination of RmAFP1 would be 0.03°C/min. This value is one third of the lowest heating rate of 0.1°C/min achievable on the used DSC and could explain the higher obtained T_m of RmAFP1 with DSC than with CD spectroscopy.

Even though CD spectroscopy has the advantage of circumventing the dependence of T_m on the rate of heating, it still has a potentially significant downside; the sample must be allowed time to reach equilibrium at all measured temperatures, thus exposing the protein to high temperatures for extended periods of time, increasing the risk of irreversible transitions. However, this does not seem to occur in the case for RmAFP1 thus enabling us to obtain sufficient data from which to determine the T_m (Fig. 2).

The T_m of RmAFP1 of 28.5°C is the lowest reported so far for insect AFPs, and is among the lowest reported T_m for proteins[30–33] although it has been observed that *C. perlata* AFPs loses their activity after 2 h at 22°C.[14] This is in contrast to other insect AFPs[12,21,22] where T_m above 60°C are reported. These AFPs are rich in disulfide bridges (7–8), which is most likely the dominant contributing factor to their heat stability.[34] RmAFP1 has only one disulfide bridge and the *C. perlata* AFP has none.[14] Furthermore, a type I fish AFP, with an α-helical structure and no cysteines, have been reported to have a T_m of only 18°C.[35]

Two different "strategies" for obtaining heat tolerant insect AFPs have been observed—high melting temperatures or great refolding abilities. As illustrated in Eq. (1) both of these qualities would limit the formation of the irreversibly denatured, I, form. The high heat tolerance of AFPs could be related to other functions than freeze avoidance. For example, it has been demonstrated that some AFP isoforms of *M. punctipennis* are expressed in the summer, and that mRNA levels of these are increased upon heat exposure.[36] Furthermore, these AFPs had a positive effect on the growth of bacteria upon exposures of 40°C and on yeast during exposures of 50°C, indicating some kind of protection against heat-related injury. Additionally, these AFPs also showed thermal protective effects at the protein level, as their presence gave rise to a smaller loss of activity of the enzyme lactate dehydrogenase upon heat exposure at 65°C for 1 h.[36] However, it is not expected that RmAFP1 have the same heat stabilizing attributes as these temperatures are above its T_m, although antifreeze activity have been found in *R. mordax* tissue during summer,[8] it was not established if this was evoked by the RmAFP1 isoform examined here.

Refolding capabilities and refolding rates of RmAFP1

The ΔH_{unfold} of RmAFP1 decreases by only 1% point per heat cycle to 60°C, indicating a near complete refolding of the protein. A high refolding capacity has also been observed for other AFPs.[13,19–22] In two cases the activity of the protein was measured to ensure that the protein refolded to its native state, and only one of these showed that the protein (DAFP-4) had retained most of its activity. Here, we also show that the RmAFP1 has activity almost similar to the untreated control after subjection to 10 heat cycles to both 60 and 70°C. When the protein sample was repeatedly heated to 80°C the ΔH_{unfold} decreased by 6.5% of the initial value per run (Fig. 3). This indicates that the heat damage at 80°C is much more extensive than at 60 and 70°C, though the loss is still low compared with other proteins in general. The sample heated repeatedly to 80°C seemed to have undergone further refolding in the time period prior to the activity was measured as it showed a higher antifreeze activity than expected from the reduction in ΔH. A reason for this could be that the equilibrating time of 45 min at 1°C between the cycles to 80°C is insufficient to obtain equilibrium, maybe because of more severe damage obtained at 80°C. The basis of this could be that breakage of the disulfide bridge had occurred at 80°C and that this bridge was not fully restored[37,38] within the 45 min equilibrating time, but have had time to undergo further refolding before the antifreeze activity measurements were performed. Another possibility is that the exposure and binding to ice during the antifreeze activity measurement have contributed to the refolding process. Further, insight into the refolding process is needed to elucidate the extent to which the proteins retain the capacity to recover after exposures to 80°C.

Apparent negative activation energy for protein folding has been observed previously and is associated with temperature-dependent changes of either hydration or ground state conformation [i.e., "U" in Eq. (1)].[39] If, for example, the unfolded (U) state attains a (average) conformation upon heating that fold through a less favourable path, this may retard the process even though the kinetic energy increases. Figure 5 shows that as the temperature is lowered to 1°C, the refolding rate begins to stagnate. Thus, at temperatures below 1°C the rate of refolding could decrease, that is, show positive activation energy.

If we consider the protein stability expressed as $\Delta G^0 = -RT \times \ln(k_+/k_-)$ or $\Delta G^0 = \Delta H - T \times \Delta S$ around 0°C, where the AFP is biologically active,

this work suggest a ΔG^0 of about 20–25 kJ/mol for RmAFP1. This is quite similar to that obtained for a fish type III AFP at 0°C,[13] and is in the low range compared to proteins in general.[40] The comparably low stabilities of these AFPs may suggest that they retain a relatively high flexibility around 0°C where they exert their activity. AFPs with broad ice binding motifs have a tendency to have fewer disulfide bridges than AFPs with narrow ice binding motifs, thus also indicating more flexibility. Furthermore, it has been observed that AFPs from *D. canadensis* change conformation upon ice binding, despite being rather rigid due to disulfide bonds.[41] We speculate that the binding to ice requires some flexibility of the protein, and that this flexibility may be needed in a higher extent for AFPs with broad ice binding motifs as interaction with a larger ice surface has to be made.

Although the disulfide bridges give a higher thermal stability, their presence is a distinct disadvantage when it comes to expressing the proteins in prokaryotes. This is often necessary if large quantities are to be produced, and thus a disadvantage in a technological point of view. The RmAFP1 is the first reported insect AFP to have both a low number of disulphide bridges (only one) and at the same time be tolerant to high temperature exposures.

Despite the insect AFPs' similar overall tertiary structure consisting of a β-helix with a flat threonine-rich ice interacting side, their reported stabilities are quite diverse. However, most of them seem to withstand great heat exposure, which is a peculiar ability considering their native function. This could indicate that the proteins have some additional functions, as observed for MpAFP.[36] The AFP1 from *R. mordax* have an unusual stability profile. Thus, the protein is thermodynamically quite heat labile and has a remarkably low T_m of 28.5°C at pH 7.4. The transitions between folded and unfolded states are slow, and show apparent negative activation energy of refolding in the studied temperature interval of 1–20°C, which may reflect temperature dependent changes in the folding pathway. In contrast to the marginal thermodynamic stability, the protein shows a conspicuous colloidal stability, as the majority of the activity is regained after repeated heat cycles up to 70°C, that is, virtually all protein returns to the folded state following cooling. This almost complete refolding is an interesting property and makes RmAFP1 promising candidate for use in industrial purposes where inactivation following heat exposure could otherwise be a potential problem.

Materials and Methods

Preparation of RmAFP1

Recombinant RmAFP1 (12,543 Da) was obtained as previously described,[42] although omitting the ion exchange purification step. The lyophilized untagged RmAFP1 was dissolved in a potassium-free phosphate buffered saline (137 m*M* NaCl, 10 m*M* Na_2HPO_4, pH 7.4) to a concentration of 0.25 mg/mL, divided into small aliquots and stored at −18°C until used. The protein concentration was determined using the BCA assay standardized to a quantitative amino acid analysis (Department of System Biology, DTU, Kgs Lyngby, Denmark).

DSC analysis

Thermal profiles of the protein were obtained using a DSC (N-DSC III model 6300, TA Instruments, New Castle, DE), holding 300 μL sample. The samples were degassed for 10 min prior to loading and scanned at 4 atmospheres of pressure. Corresponding blanks were subtracted from all runs. The experimental reproducibility was improved by an initial heating/cooling cycle to 40°C at 2°C/min, which was used throughout the DSC measurements. The exact cause for this improvement is not known, but it may rely on uniform thermal histories of the samples. The DSC output was processed with the software NanoAnalyze (TA Instruments, New Castle, DE). An average for ΔH_{unfold} was obtained from six heating scans. The apparent melting temperatures of the protein, T_{mApp}, were assessed by heat cycles from 10 to 60°C at different heating rates in the interval 0.1–2.0°C/min. The refolding abilities of RmAFP1 were assessed by comparing ΔH of 10 successive heat cycles from 1 to 60°C, 1 to 70°C, and 1 to 80°C, respectively. A cooling rate of 10°C/min was initiated once the samples reached the maximum temperature. This was followed by a 45 min equilibration step at 1°C. Following the calorimetric measurements, the samples were retrieved from the DSC, and stored at −18°C for subsequent antifreeze activity measurements.

CD spectroscopy

The configuration of the protein was monitored using a Jasco J-715 circular dichrometer equipped with a thermostat accessory. Samples were measured in the far UV spectrum (200–250 nm) using a 0.1 cm path-length quarts cuvette holding 220 μL of sample. All measurements were performed at a scan rate of 50 nm/min and a constant N_2-flow. To assess the protein refolding rates, samples were heated to 50°C for 12 min to denature the protein prior to cooling to the respective measuring temperatures, where the spectra were continuously recorded to monitor the refolding process.

To determine the T_m of RmAFP1, CD spectra were obtained at 10 different temperatures in the interval 1–68°C. To ensure that the protein had reached structural equilibrium, all samples were held at each temperature for 45 min prior to CD measurements. The measurements were taken in

duplicates at each temperature. As the CD spectrum of the folded protein has its global minimum at 218 nm,[42] here referred to as $mdeg_{218}$, the CD value at this wavelength, rather than the entire spectrum, was used to monitor temperature-induced changes in protein configuration. The recorded changes were fitted to the equation

$$F_U(T) = \frac{L}{1 + ae^{-bT}} \quad (6)$$

describing a sigmoid curve, where F_U is the fraction of unfolded protein, T is the temperature in degree Celsius and Liter is the saturation value, here set to 1, as this represents complete unfolding of the protein. The T_m was obtained from Eq (6) as it by convention is defined as the temperature where half of the protein population is unfolded, that is, where $F_U(T) = 0.5$, provided that the aggregation is slow (as was the case for RmAFP1).

Antifreeze activity measurement

The antifreeze activity was measured using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY), as described elsewhere.[42]

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